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2.0 BG1Luc ER TA Protocol Components

2.1 Overview

The BG1Luc ER TA utilizes an ER responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line, BG-1, to detect substances with *in vitro* ER agonist or antagonist activity. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. A concentration-response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance. The advantages of using a luciferase reporter gene system are low background, high sensitivity, rapidity, and a wide dynamic range.

The primary objective of this test method is to provide a qualitative assessment of *in vitro* estrogenic activity (i.e., a substance is either positive or negative for estrogenic activity), but quantitative analysis is also performed to provide additional information on the estrogenic potency of test substances (e.g., EC_{50}). Separate protocols are used to identify substances possessing either ER agonist or antagonist activity, although the two protocols share most major components.

ICCVAM previously recommended minimum essential test method components for *in vitro* ER TA protocols (ICCVAM 2003), which included the following considerations:

- A reference standard should be included to demonstrate the adequacy of the test method for detecting ER agonists or antagonists.
- In each study, a set of concurrent solvent controls should be included.
- An evaluation of cytotoxicity should be included in each study.
- A weak positive agonist control with a half-maximal effective concentration (EC_{50}) two to three orders of magnitude higher than the reference estrogen should be included in each study to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak estrogen agonists.
- A weak positive antagonist control, that inhibits the reference estrogen response by 50% (IC_{50}) at a concentration two to three orders of magnitude higher than the reference anti-estrogen, should be included in each study to provide a measure by which to demonstrate that the test method is functioning properly and is sufficiently sensitive to detect weak estrogen antagonists.

- The maximum test substance concentration should be 1 mM unless otherwise limited by solubility or cytotoxicity.
- A minimum of seven concentrations spaced at logarithmic (log10) intervals, up to the limit concentration, should be tested.
- EC_{50} or IC_{50} values should be calculated for all positive substances when possible.
- Protocols should contain established test plate acceptance criteria.

The ICCVAM-recommended test method components were incorporated into the BG1Luc ER TA protocols during a protocol standardization study coordinated by NICEATM and conducted at XDS (**Annex C**). The goal of the standardization study, in which eight agonists and eight antagonists were tested, was to develop protocols for use in the ICCVAM-sponsored international validation study. Once the multi-phase validation study was initiated, the protocols continued to be refined after each phase resulting in optimized protocols for agonist and antagonist testing (see **Annexes E** and **F**, respectively). The remainder of this section provides details on the essential test method components and the rational for their inclusion in the optimized protocols.

2.2 Materials

2.2.1 BG1Luc4E2 Cells

The BG-1 cell line, derived from immortalized human ovarian adenocarcinoma cells, has been used extensively to examine the estrogenic effects of chemicals (Park et al. 2009; Pujol et al. 1998; Rogers and Denison 2000; Zhou et al. 2005). BG-1 cells endogenously express both human ERα and ERβ and (Wong and Matsumura 2006), although ERα is the predominate isoform (90%) (Monje and Boland 2001; Pujol et al. 1998). BG-1 cells were stably transfected with a plasmid containing a firefly luciferase reporter gene under control of four estrogen response elements placed upstream of the mouse mammary tumor viral (MMTV) promoter. The resultant BG1Luc4E2 cell line expresses luciferase activity in response to estrogen and estrogen-like substances. While the MMTV promoter sequence used for the BG-1 plasmid construct reportedly lacks the glucocorticoid-responsive elements normally present in this region (Rogers and Denison 2000), the BG1Luc ER TA developers examined possible cross-reactivity with other steroid and non-steroid hormones. Progesterone, testosterone, all-trans retinoic acid, and thyroid hormone did not induce luciferase activity, while dihydrotestosterone (AR ligand) and dexamethasone (GR ligand) caused only a small degree of induction (Rogers and Denison 2000). Together, these results indicate that the BG1Luc4E2 cells exhibit only minor cross-reactivity with other ligands for members of the nuclear hormone receptor superfamily.

Cryopreserved BG1Luc4E2 cells from the cell bank established at XDS were provided to ECVAM and Hiyoshi for conduct of the validation study. ECVAM and Hiyoshi propagated and cryopreserved multiple ampoules of cells to establish their working cell banks for use throughout the study.

2.2.2 Cell Culture Reagents and Supplies

The BG1Luc ER TA requires general cell culture materials, reagents, and supplies (see **Annexes E** and **F** [protocols] for formulations, and concentrations of solutions and media). The participating laboratories independently acquired cell culture materials, reagents, and supplies.

The following reagents are used for cell culture procedures in the BG1Luc ER TA:

- Dimethyl sulfoxide (DMSO)
- Luciferase reagent
- PBS
- Trypsin (2.5% v/v in PBS as a cell dissociation agent)
- Gentimicin (G418)
- RPMI 1640 media, containing L-glutamine and supplemented with Penicillin-Streptomycin (Pen-Strep), and FBS is used for thawing and freezing of cells, and maintenance tissue culture.
- Dulbecco's Modification of Eagle's Medium (DMEM) containing high glucose (4.5 g/L) and sodium pyruvate, without phenol red. DMEM is supplemented with charcoal/dextran treated (to remove free hormones from sera) fetal bovine sera (FBS), Pen-Strep, and L-glutamine, which is designated as estrogen free media (EFM). Cells are transferred from RPMI to EFM prior to testing.

Required laboratory cell culture supplies include the following:

- Cryogenic ampoules (cryovial)
- Plastic culture tubes (e.g., 50 mL conical tubes)
- Hemocytometer
- Pipettes, pipetters, repeat pipetters, pipette tips
- Sterile, disposable tissue culture plasticware (e.g., 25 cm^2, 75 cm^2, 96-well microtiter plates)

2.2.3 Equipment

Performance of the BG1Luc ER TA requires a laboratory equipped with a designated cell culture area. Essential equipment required for the conduct of the test method includes:

- Analytical balance
- Biological safety hood, class II or higher with HEPA filter
- Centrifuge (capable of 1000 x g)
- 4 °C Refrigerator
- Freezers, -20 °C, and -70 °C
- Incubator (37 °C ± 1 °C, 90% ± 5% humidity, and 5% ± 1% CO_2/air)
- Liquid nitrogen cryostorage
- Microplate, auto-injecting luminometer
- Shaker for 96-well plates
- Vortex mixer

2.3 Cell Culture

The primary objective for any tissue culture operation is to maintain consistency in the cultures used. To do this, strict control of culture conditions (i.e. growth and maintenance media, culture schedules, culture flasks and plates, substrate type, seeding conditions, dissociation methods) must be maintained. Strict control must also be taken to properly freeze, maintain, and thaw cultures in a systematic manner, since cryopreservation techniques can affect subsequent culture growth and performance. All pertinent information about cell culture reagents and supplies (e.g., lot number, manufacturer, product code, certificates of analysis) should be collected and maintained in log books and reports.

Cryopreserved BG1Luc4E2 cells are thawed, re-suspended in RPMI media, transferred into 25 cm^2 tissue culture flasks, and incubated at 37 °C ± 1 °C, 90% ± 5% humidity, and 5% ± 1% CO_2/air for 48 to 72 hours. When cells reach 80 to 90% confluence (as estimated from a visual inspection of cell density), they are removed from the flask by trypsinization. A dissociated single-cell suspension is added to new flasks for propagation and the cells are passaged/subcultured at least two times before conditioning in EFM. At 48 to 72 hours after the second subculture, cells are trypsinized, pelleted, and the RPMI media removed. Cells are then resuspended in EFM and the cell suspension added to new flasks for conditioning. At this time, gentamicin (G418) is added to the EFM to select BG1Luc4E2 cells containing the G418 resistant reporter plasmid. When cells are 80% to 90% confluent, they are trypsinized, counted, and seeded into 96-well plates for testing.

2.4 Reference Standards and Controls

ICCVAM (2003) recommends the use of appropriate reference standards and controls for ER TA test methods in order to maximize test method intra- and inter-laboratory reproducibility and minimize the likelihood of erroneous results.

2.4.1 Vehicle Control

- *1% DMSO in EFM is used as the concurrent vehicle control for all testing in both Agonist and Antagonist protocols.*

A concurrent vehicle control in ER TA agonist and antagonist test methods provides a measure of the extent of TA in the absence of the reference standard, control or test substances. For ER TA test methods, the concurrent vehicle control is the baseline against which the extent of TA induction is determined (in the BG1Luc ER TA averaged test plate vehicle control relative light unit [RLU] values are subtracted from test substance, reference standard, and control RLU values). XDS tested several solvents when developing the BG1Luc ER TA, and selected a solution of 1% DMSO (v/v) in EFM because of its ability to solubilize a wide range of both hydrophilic and hydrophobic substances and to achieve relatively high concentrations of test substance without reducing cell viability.

2.4.2 Estrogenic Reference Standard

In accordance with the ICCVAM (2003) recommendations, 17β-estradiol (E2, CASRN 50-28-2) is used as the reference estrogen to demonstrate the adequacy of the ER TA test method. In the BG1Luc ER TA, this is based on the ability of the E2 reference standard to induce ER TA activity.

The concentrations of E2 used in different phases of testing are provided in **Table 2-2**. A 4-point dilution was used in ER agonist range finder testing to broadly define the E2 curve response in terms of bottom, slope, and top. An 11-point dilution of E2 was then used in comprehensive ER agonist testing to more fully define the E2 response curve, thereby allowing an EC_{50} to be calculated. E2 was used at a concentration of 9.18×10^{-11} M in ER antagonist range finder and comprehensive testing in order to provide a level of induction against which antagonistic effects of test substances could be assessed.

Table 2-2 E2 Concentrations Tested

E2 Concentration (M)	Agonist Test Method		Antagonist Test Method	
	Comprehensive Testing	Range Finder Testing	Comprehensive Testing	Range Finder Testing
3.67×10^{-10}	X	-	-	-
1.84×10^{-10}	X	X	-	-
9.18×10^{-11}	X	-	X	X
4.59×10^{-11}	X	X	-	-
2.29×10^{-11}	X	-	-	-
1.15×10^{-11}	X	X	-	-
5.73×10^{-12}	X	-	-	-
2.87×10^{-12}	X	X	-	-
1.44×10^{-12}	X	-	-	-
7.16×10^{-13}	X	-	-	-
3.59×10^{-13}	X	-	-	-

Abbreviations: E2= 17β-estradiol

2.4.3 Weak Agonist Control

- *p,p' Methoxychlor (Meth, CASRN 72-43-5) is used as the weak positive control.*

ICCVAM (2003) recommends that a positive control with a half-maximal effective concentration (EC_{50}) two to three orders of magnitude higher than E2 (EC_{50} = 3 x 10^{-12} M) be included in each study to demonstrate that the test method is functioning properly and is sufficiently sensitive for detecting weak estrogen agonists. However, given that the range of responses expected to be assessed with this method was greater than 6 orders of magnitude, the SMT concluded that a positive control with a higher EC_{50} multiple would be more appropriate. During protocol standardization, a number of substances were evaluated for use as the weak agonist control (**Annex C**). Based on this evaluation, *p,p'*-methoxychlor (CASRN 72-43-5) was considered to be the most appropriate control because it produced the most consistent ER TA response curves in the desired range (EC_{50} = 6 μM), approximately 6 orders of magnitude higher than E2 (EC_{50} = 3 x 10^{-12} M in BG1Luc ER TA). A methoxychlor concentration of 9.06×10^{-6} M was selected because this is the lowest concentration giving a maximum response**.**

2.4.4 Anti-Estrogenic Reference Standard

- *Raloxifene HCL (Ral, CASRN 82640-04-8 is used as the anti-estrogenic reference standard.*

Although ICCVAM (2003) recommends ICI 182,780 as a reference standard in ER TA antagonist assays, this substance has limited commercial availability (ICCVAM 2006). As an alternative, Ral, a strong estrogen antagonist that is listed as a recommended reference substance in ICCVAM (2003), was evaluated for use as the reference standard during the protocol standardization study. Results indicated that Ral consistently produced full concentration response curves with a mean IC_{50} value of 2.24×10^{-9} M in BG1Luc ER TA (**Annex C**), and was therefore selected as the anti-estrogenic reference standard for the validation study.

The concentrations of Ral used in ER antagonist range finder and comprehensive testing are provided in **Table 2-3**. A 3-point dilution was used in ER Antagonist range finder testing to broadly define the top, slope, and bottom of the Ral response curve. A 9-point dilution of Ral was then used in comprehensive ER antagonist testing to more fully define the Ral response curve, thereby allowing the calculation of an IC_{50}.

Table 2-3 Raloxifene Standard Concentrations Tested

Raloxifene Concentration (M)	Antagonist Comprehensive Testing	Antagonist Range Finder Testing
2.45×10^{-8}	X	-
1.23×10^{-8}	X	-
6.14×10^{-9}	X	-
3.06×10^{-9}	X	X
1.53×10^{-9}	X	-
7.67×10^{-10}	X	X
3.82×10^{-10}	X	-
1.92×10^{-10}	X	X
9.57×10^{-11}	X	-

2.4.5 Weak Antagonist Control

- *Tamoxifen (Tam, CASRN 10540-29-1) is used as the weak positive control for antagonist comprehensive testing.*

The use of a weak antagonist as a concurrent control in ER TA antagonist test methods provides a measure of the range of responses that can be detected by the test. ICCVAM (2003) recommends using a

weak positive control with an IC_{50} at least three orders of magnitude higher than the reference antagonist, Ral (IC_{50}= 2.24 x 10^{-9}M). During protocol standardization (see **Annex C**), a number of substances were evaluated for use as the weak antagonist control. Flavone produced a dose response and an IC_{50} = 4.3 x 10^{-7}M, which was consistent with the single literature reference for this compound (reported IC_{50}= ~15 μM) and was two times below that of Ral, Based on these results, flavone was chosen as the weak antagonist control for the validation study. However, after reviewing data from the completed study, it became apparent that the vast majority of test substances classified as "negative" or "presumed negative" produced a "positive" response at concentrations above ~ 10μM (see **Annex K** for ER TA antagonist testing results), and the use of flavone as a weak antagonist control was therefore reconsidered, as discussed below.

The antagonist method is a "loss of function" method where a positive result is based on a *decrease* in luciferase activity (in contrast to the agonist method in which an *increase* in luciferase activity [i.e., "gain of function"] is indicative of a positive response. Consequently, any substance that disturbs cellular homeostasis or causes cytotoxicity will produce an apparent "positive" response (i.e., dead cells produce no signal, and therefore produce the maximum response). To account for this, an assessment of cell viability is included in the agonist and antagonist test method protocols (**Section 2.6**). Data from antagonist validation testing were reviewed to determine if there was a correlation between the observed decrease in luciferase activity (positive response) and a loss in cellular viability. In many cases, there was no observed decrease in cellular viability at the highest concentration tested. In cases where a loss of viability was observed, a decrease in luciferase activity usually *preceded* a loss of cellular viability, sometimes at concentrations up to two or three log dilutions lower than the cytotoxic concentration.

These findings indicate that cellular viability cannot be reliably used as an indicator of test substance interference with the BG1Luc ER TA, and that it is not possible to distinguish true positives from false positives at concentrations above ~ 10μM. In addition, ICCVAM could not identify in the literature any substances classified as "positive" for ER antagonism with an IC_{50} > 10μM. Therefore, the SMT established 10 μM as the upper limit of utility for determining antagonist activity in the BG1Luc ER TA. Because the 10 μM would preclude the use of flavone as a weak antagonist control (IC_{50} = 15μM), tamoxifen was selected by the SMT as a weak antagonist control, since it has been conclusively shown to bind the ER (46/46 studies, **Table 3-2**) and act as an ER antagonist in most ER TA studies (20/22 studies, **Table 3-2**). The mean IC_{50} for tamoxifen in ER TA studies is 7.20 x 10^{-7}M, which is two-fold above that of Ral yet below the 10 μM upper limit of the assay.

2.5 Test Substance Concentrations

- For agonist testing: the highest soluble, non-cytotoxic concentration should be tested up to a limit of 1 mM.
- For antagonist testing: the highest soluble, non-cytotoxic concentration should be tested up to a limit of 10μM.

ICCVAM (2003) recommends that the maximum test substance concentration should be 1 mM unless otherwise limited by solubility or cytotoxicity (note: reference substances were coded in order conduct the validation study in a blinded manner, therefore the participating laboratories were instructed to use mg/mL as the limit concentration). However, as outlined above in **Section 2.4.5**, the BG1Luc ER TA validation study data indicate that concentrations above 10μM in the antagonist assay consistently produce false "positive" responses in this loss-of-function assay**.** Consequently, the SMT established 10 μM as the upper limit of utility for determining antagonist activity in the BG1Luc ER TA.

2.5.1 Solubility Testing

- The starting concentration for range finder testing is established by determining the maximum test substance solubility in 100% DMSO in EFM.

ICCVAM (2003) recommends that the maximum test substance concentration should be 1 mM unless limited by solubility or cytotoxicity. Procedures used to assess solubility are provided in this section and procedures used to assess Cytotoxicity are provided below in **Section 2.5.2**.

During Phase 1 and Phase 2 testing, maximum test substance solubilities were determined at log intervals up to 1 mg/mL (v/v in 1% DMSO/cell culture media). Following Phase 2 of the validation study, a high degree of variability was noted in solubility assessment performed on the same substance across different labs. Problems associated with log scale dilutions in the 1% DMSO medium were believed to be causing the variability. To reduce differences in solubility estimates between labs, protocols were modified to use test substance solubility in 100% DMSO as the starting concentration for range finder testing. This protocol modification was used for Phase 3 and 4 testing. Test substance solubility data are provided in **Section 4.**

2.5.2 Cytotoxicity Testing

- A qualitative visual observation method that assesses viability on a scale of 1 (normal) to 4 (significant loss of viability) is used to assess cell viability in the BG1Luc ER TA. Viability scores of 2 or greater are classified as cytotoxic.

- The assessment of cytotoxicity is incorporated into agonist and antagonist range finder testing, as described below in **Sections 2.6.1** and **2.6.2** respectively.

An assessment of cell viability was recommended by the Panel (ICCVAM 2002a, 2002b) to help define the upper limit for test substance concentrations, similar to the Maximum Tolerated Dose (MTD) approach used in *in vivo* studies. During the BG1Luc ER TA protocol standardization study (**Annex C**), XDS used the CellTiter-Glo® (Promega Corporation) quantitative cell viability assay to assess the viability of BG1Luc4E2 cells following exposure to increasing concentrations of test substance. Cell-Titer-Glo® measures cell viability via a luminescent signal that is proportional to the amount of ATP in viable cells. Results indicated that the ER TA activity of the fixed amount of E2 used in antagonist testing was significantly reduced when the reduction in ATP level per well exceeded 20%. Based on these results, concentrations of substance that reduced cell viability more than 20% were classified as cytotoxic. However, like the BG1Luc ER TA, the CellTiter-Glo® assay is based on a luminescent endpoint (ER TA luciferase vs. ATP luminescence). For this reason, the use of parallel plates is necessary because ATP luminescence cannot be delineated from ER TA associated luciferase activity.

Therefore, an alternative qualitative method to assess cell viability was developed by XDS during the protocol standardization study (**Annex C**). This method relies on visual observation of cell density and morphology to assign cell viability scores using criteria listed in **Table 2-4.** Test substance concentrations of two or greater are considered to be cytotoxic.

A direct comparison of the CellTiter-Glo® assay and visual observation methods indicated that CellTiter-Glo® values of 80% or greater corresponded with a viability score of 1 in the visual observation method study (**Annex C**). Therefore, the visual observation method was considered adequate for assessing cell viability in the BG1Luc ER TA, thereby precluding the need for parallel test plates.

Table 2-4 Visual Observation Scoring Table

Viability Score	Brief Description
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
P	Wells containing precipitation are to be noted with “P”

2.6 Range Finder Testing

The purpose of range finder testing is to establish the concentration range of a test substance to be included in comprehensive testing. This involves identifying both an appropriate starting concentration and dilution scheme. The starting concentration of a test substance is based on the highest soluble concentration that is not cytotoxic, as described in **Section 2.5**. Based on the results from range finder testing, either a 1:5 or 1:2 dilution scheme will be selected for comprehensive testing; a 1:5 dilution covers a wider concentration range (7.5 log dilutions), while a 1:2 dilution provides higher resolution over a smaller range (3.5 log dilutions). Procedures for range finder testing along with the criteria used to determine the appropriate testing range are provided below.

2.6.1 Agonist Range Finder Testing

2.6.1.1 Reference Standard and Control Concentrations used for Agonist Range Finder Testing.

- E2, the reference estrogen, is run in duplicate at four concentrations (1.84×10^{-10}, 4.59×10^{-11}, 1.15×10^{-11} and 2.87×10^{-12} M)
- The Vehicle control (1% DMSO v/v in EFM) is run in quadruplicate

2.6.1.2 Agonist Range Finder Plate Design

- All 96 wells of the test plate are used during range finder testing, thereby allowing a maximum of six substances tested at seven concentrations in duplicate on each range finder plate using starting concentrations that were determined during solubility testing. Plate design for agonist testing is provided below in **Figure 2-4.**

In Phase 1 of the validation study, the lead laboratory (XDS) conducted studies to optimize the plate design in order to improve the statistical power and to allow all 96 wells to be utilized (**Annex M**). Results demonstrated that, although there were statistically significant differences in values between outside and inside wells, the differences did not affect the selection of the appropriate starting concentrations for comprehensive testing (see **Annex M**). Therefore, the design of agonist and antagonist range finding plates was modified to use all 96 wells of the test plate, with six test substances being tested at seven concentrations in duplicate on each range finder plate.

Figure 2-4 Plate Layout for Agonist Range Finder Testing

TS1-1	TS1-1	TS2-1	TS2-1	TS3-1	TS3-1	TS4-1	TS4-1	TS5-1	TS5-1	TS6-1	TS6-1
TS1-2	TS1-2	TS2-2	TS2-2	TS3-2	TS3-2	TS4-2	TS4-2	TS5-2	TS5-2	TS6-2	TS6-2
TS1-3	TS1-3	TS2-3	TS2-3	TS3-3	TS3-3	TS4-3	TS4-3	TS5-3	TS5-3	TS6-3	TS6-3
TS1-4	TS1-4	TS2-4	TS2-4	TS3-4	TS3-4	TS4-4	TS4-4	TS5-4	TS5-4	TS6-4	TS6-4
TS1-5	TS1-5	TS2-5	TS2-5	TS3-5	TS3-5	TS4-5	TS4-5	TS5-5	TS5-5	TS6-5	TS6-5
TS1-6	TS1-6	TS2-6	TS2-6	TS3-6	TS3-6	TS4-6	TS4-6	TS5-6	TS5-6	TS6-6	TS6-6
TS1-7	TS1-7	TS2-7	TS2-7	TS3-7	TS3-7	TS4-7	TS4-7	TS5-7	TS5-7	TS6-7	TS6-7
E2-1	E2-2	E2-3	E2-4	VC	VC	VC	VC	E2-1	E2-2	E2-3	E2-4

Abbreviations: E2-1 to E2-4 = concentrations of the E2 reference standard (from high to low); TS1-1 to TS1-7 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-7 = concentrations (from high to low) of test substance 2 (TS2); TS3-1 to TS3-7 = concentrations (from high to low) of test substance 3 (TS3); TS4-1 to TS4-7 = concentrations (from high to low) of test substance 4 (TS4); TS5-1 to TS5-7 = concentrations (from high to low) of test substance 5 (TS5); TS6-1 to TS6-7 = concentrations (from high to low) of test substance 2 (TS2); VC = vehicle control (DMSO [1% v/v EFM.]).

2.6.1.3 Agonist Range Finder Plate Acceptance Criteria

- The mean DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control mean RLU value.
- E2 Induction must be greater than three-fold. Induction is calculated by averaging the highest E2 reference RLU values from each E2 concentration curve and then dividing this by the average DMSO control RLU value.

Data from plates that fail any acceptance criterion should be discarded as inadequate and the experiment repeated.

2.6.1.4 Interpretation of Results from Agonist Ranger Finder Testing

- If no points on the test substance concentration curve are greater than the DMSO control mean plus three times its standard deviation (SD), comprehensive testing for ER agonist activity should be conducted using the highest non-cytotoxic concentration tested.
- If any points on the test substance concentration curve are greater than the DMSO control mean plus three times its SD, testing should use a starting concentration one log higher than the concentration giving the highest adjusted RLU value.
- An 11-point 1:2 serial dilution (covering approximately three orders of magnitude), should be used if the resulting concentration range will resolve the full dose response curve of the

test substance, as estimated from the range finder data. Otherwise, an 11-point 1:5 dilution is used.

- If a substance exhibits a biphasic concentration response curve (hormetic or U-shaped) not associated with cytotoxicity in the range finder test, an attempt should be made to resolve the complete shape of both phases of the curve in comprehensive testing by using a 1:5 serial dilution starting with a concentration one log higher than the concentration associated with the peak of activity closest to the high end of the concentration range tested.

2.6.2 Antagonist Range Finder Testing

2.6.2.1 Reference Standard and Control Concentrations used for Antagonist Range Finder Testing.

- A single concentration of E2 (9.18×10^{-11} M), intended to provide 80% of the maximum E2 induction, is run in triplicate.
- Three concentrations of the reference anti-estrogen, Raloxifene HCl (3.06×10^{-9}, 7.67×10^{-10}, and 1.92×10^{-10} M) are each run in duplicate.
- The vehicle control (1% DMSO v/v in EFM) is run in triplicate.
- All reference anti-estrogen and test wells contain a fixed concentration of E2 (9.18×10^{-11} M), intended to provide 80% of the maximum E2 induction.

2.6.2.2 Antagonist Range Finder Plate Design

All 96 wells of the test plate are used during range finder testing, thereby allowing a maximum of six substances tested at seven concentrations in duplicate on each range finder plate using starting concentrations that were determined during solubility testing. The plate design for antagonist testing is provided in **Figure 2-5.**

Figure 2-5 Plate Layout for Antagonist Range Finder Testing

TS1-1	TS1-1	TS2-1	TS2-1	TS3-1	TS3-1	TS4-1	TS4-1	TS5-1	TS5-1	TS6-1	TS6-1
TS1-2	TS1-2	TS2-2	TS2-2	TS3-2	TS3-2	TS4-2	TS4-2	TS5-2	TS5-2	TS6-2	TS6-2
TS1-3	TS1-3	TS2-3	TS2-3	TS3-3	TS3-3	TS4-3	TS4-3	TS5-3	TS5-3	TS6-3	TS6-3
TS1-4	TS1-4	TS2-4	TS2-4	TS3-4	TS3-4	TS4-4	TS4-4	TS5-4	TS5-4	TS6-4	TS6-4
TS1-5	TS1-5	TS2-5	TS2-5	TS3-5	TS3-5	TS4-5	TS4-5	TS5-5	TS5-5	TS6-5	TS6-5
TS1-6	TS1-6	TS2-6	TS2-6	TS3-6	TS3-6	TS4-6	TS4-6	TS5-6	TS5-6	TS6-6	TS6-6
TS1-7	TS1-7	TS2-7	TS2-7	TS3-7	TS3-7	TS4-7	TS4-7	TS5-7	TS5-7	TS6-7	TS6-7
Ral-1	Ral-2	Ral-3	VC	VC	VC	E2	E2	E2	Ral-1	Ral-2	Ral-3

Abbreviations: E2 = E2 control; Ral-1 to Ral-3 = concentrations of the Raloxifene/E2 reference standard (from high to low); TS1-1 to TS1-7 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-7 = concentrations (from high to low) of test substance 2 (TS2); TS3-1 to TS3-7 = concentrations (from high to low) of test substance 3 (TS3); TS4-1 to TS4-7 = concentrations (from high to low) of test substance 4 (TS4); TS5-1 to TS5-7 = concentrations (from high to low) of test substance 5 (TS5); TS6-1 to TS6-7 = concentrations (from high to low) of test substance 6 (TS6); VC = vehicle control (DMSO [1% v/v EFM.]).

2.6.2.3 Antagonist Range Finder Plate Acceptance Criteria

- The mean DMSO control RLU value for each plate must be within 2.5 times the SD of the historical DMSO control mean RLU value.
- Test plate E2 control RLU values must be within 2.5 times the SD of the historical E2 control mean RLU value.
- Plate reduction must be greater than three-fold. Reduction is calculated by dividing the averaged highest Ral reference RLU value by the averaged lowest Ral RLU value.

Data from plates that fail any acceptance criterion should be discarded and the experiment repeated.

2.6.2.4 Interpretation of Results from Antagonist Ranger Finder Testing

- If no points on the test substance concentration curve are less than the mean of the DMSO control minus three times the SD, comprehensive testing for ER antagonist activity should be conducted using the highest non-cytotoxic concentration tested.
- If any points on the test substance concentration curve are less than the DMSO control mean minus three times the SD, testing should use a starting concentration one log higher than the concentration giving the lowest adjusted RLU value.
- An 11-point 1:2 serial dilution (covering approximately three orders of magnitude), should be used if the resulting concentration range will resolve the full concentration response curve

of the test substance, as estimated from the range finder data. Otherwise, an 11-point 1:5 dilution is used.

- If a substance exhibits a biphasic concentration response curve (hormetic or U-shaped) not associated with cytotoxicity in the range finder test, an attempt should be made to resolve the complete shape of both phases of the curve in comprehensive testing by using a 1:5 serial dilution starting with a concentration one log higher than the concentration one log higher than the concentration associated with the lowest activity closest to the high end of the concentration range tested.

2.7 Comprehensive Testing

2.7.1 Comprehensive Agonist Testing

2.7.1.1 Reference Standard and Control Concentrations used for Agonist Comprehensive Testing.

- E2, the reference estrogen, is run in duplicate at eleven concentrations (see **Table 2-3**).
- Methoxychlor, the weak positive control, is run in quadruplicate at a single concentration of 9.06×10^{-6} M
- The vehicle control (1% DMSO in EFM) is run in quadruplicate.

2.7.1.2 Plate Design

All 96 wells of the test plate are used during comprehensive agonist testing, thereby allowing two substances tested at eleven concentrations, in triplicate, on each plate. Starting concentrations were determined during range finder testing (**Section 2.7**). Plate design for comprehensive agonist testing is provided below in **Figure 2-6.**

To evaluate the effect of using outer test plate wells on comprehensive testing, EC_{50} values from serial dilutions of BPA derived from replicates using outside wells were compared to EC_{50} values derived from replicates using inside wells. The comparisons indicated that there were no significant differences between EC_{50} values derived from replicates using outside wells and those derived from using inside wells (see **Annex M).**

Figure 2-6 Plate Layout for Comprehensive Agonist Testing

TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Meth
E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth
E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth

Abbreviations: TS1-1 to TS1-11 = concentrations (from high to low) of test substance 1; TS2-1 to TS2-11 = concentrations (from high to low) of test substance 2; E2-1 to E2-11 = concentrations of the E2 reference standard (from high to low); Meth = *p,p'* methoxychlor weak positive control; VC = DMSO (1% v/v) EFM vehicle control

2.7.1.3 Plate Acceptance Criteria for Comprehensive Agonist Testing

- The mean DMSO control RLU value for each plate must be within 2.5 times the standard deviation of the historical DMSO control mean RLU value.
- E2 Induction must be greater than three-fold. Induction is calculated by averaging the highest E2 reference RLU values from each E2 concentration curve and then dividing this by the average DMSO control RLU value.
- The E2 reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve.
- The mean plate methoxychlor control RLU value must be greater than the mean DMSO control RLU value plus three times the SD.

2.7.1.4 Modification of Plate Acceptance Criteria for Comprehensive Agonist Testing

Following Phase 2a of the validation study, the failure rates of plates used to comprehensively test four agonist substances were evaluated. The percentage of agonist test plates that failed test plate acceptance criteria across the participating laboratories was 61% (33/54) (see **Section 7, Table 7-4**). To determine if changes to test plate acceptance criteria could reduce the failure rates of comprehensive test plates without compromising the ability of the test method to detect and quantify test substance agonist or antagonist activity, a comparison was made between qualitative (i.e., positive or negative agonist classification) and quantitative (i.e., a EC_{50} value) outcomes for test plates that met all acceptance criteria

versus those that failed to meet one or more criterion (see **Section 7**, **Tables 7-5** and **7-6**). Test plate acceptance criteria based on the DMSO control RLU values and E2 reference standard minimum fold-increase induction values were not considered for modification because they are essential for monitoring background activity and reference estrogen performance. Therefore, the test plate acceptance criteria that were considered for modification were the E2 EC_{50} and methoxychlor RLU control values. Based on this evaluation, it was determined that agonist test plate acceptance criteria could be modified without compromising the ability of the test method to detect and quantify test substance agonist activity. These modifications were as follows:

- The requirement for the mean plate E2 reference standard EC_{50} value to be within 2.5 times the SD of the historical mean EC_{50} value was eliminated.
- The requirement for the mean plate methoxychlor control RLU value was changed from within 2.5 times the SD of the historical mean methoxychlor control RLU value to within 3 times the SD of the historical methoxychlor control RLU.

Changes to the agonist test plate acceptance criteria described above were used for Phase 2b, 3, and 4 testing.

2.7.1.5 Interpretation of Results from Comprehensive Agonist Testing

POSTIVE CLASSIFICATION

- All test substances classified as positive for ER agonist activity should have a concentration response curve consisting of a baseline, followed by a positive slope, and concluding in a plateau or peak. In some cases, only two of these characteristics (baseline-slope, or slope-peak) may be defined.
- The line defining the positive slope must contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the peak or first point of the plateau.
- A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the maximal value for the reference estrogen (i.e. 2000 RLU when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs).
- If possible, an EC_{50} value should be calculated for each positive substance **(Section 4)**.

NEGATIVE CLASSIFICATION

- For all concentration response curves that fail to meet the criteria for a positive response, test substances are classified as negative for agonist activity if all data points are below 20% of

the maximal value for the reference estrogen (i.e. 2000 RLU when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs).

INADEQUATE

- Data that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of activity.

2.7.1.6 New Classification Scheme

The BG1Luc ER TA is intended as part of a weight of evidence approach to help prioritize substances for ED testing *in vivo*. Part of this prioritization procedure will be the classification of the test substance as positive or negative for either ER agonist or antagonist activity. There currently are no universally accepted standards for determining whether a substance is positive for ER agonist or antagonist activity. A common approach for the classification of substances as positive is to determine the lowest effective concentration (LEC), i.e., the concentration that is significantly different from the concurrent negative control (Judson et al. 2010; Martin et al. 2010). For the protocol standardization study and all phases of testing in the BG1Luc ER TA validation study, an LEC method was used to determine whether a test substance was positive or negative. Specifically:

- A substance is considered positive for agonist activity when the average adjusted RLU for a given concentration is greater than the mean DMSO control RLU value plus three times its standard deviation (3X-SD).
- A substance is considered negative for agonist activity if the average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times its standard deviation.

This classification system appeared to work well during the protocol standardization study and during the early phases of testing (Phase1, Phase 2a, Phase 2b) and was therefore also utilized for Phase 3 and Phase 4 testing. However, the resulting data indicated that this classification scheme was resulting in an unacceptable level of "false positives" (71 out of 78 test substances were classified as positive) in the agonist assay. The contributing factors appeared to be as follows:

- The binary nature of the classification system (all substances will be classified as POS or NEG).
- Classification was based on individual values (not a curve shape), and did not accommodate high background levels or variability in test data. Consequently, single data points often

exceed the 3-X SD DMSO control line due to the variability of the test, causing substances to be classified as positive.

- Many test substances caused a significant increase in background RLUs, resulting in a baseline that was near or above the 3-X SD DMSO control and therefore causing the substances to be classified as positive.
- No allowances were made for poor quality test data (only plate acceptance criteria were considered for QC purposes)

In light of the above, the SMT agreed on a new classification scheme that addressed each of these deficiencies. These new classification criteria were applied retrospectively to all test data for the assessment of test method accuracy (**Section 5**).

2.7.2 Comprehensive Antagonist Testing

2.7.2.1 Reference Standard and Control Concentrations used for Antagonist Comprehensive Testing

- Raloxifene, the anti-estrogenic reference standard, is plated in a serial dilution consisting of nine concentrations of Ral in duplicate (see **Table 2-3**).
- A single concentration of E2 (9.18×10^{-11} M), intended to provide 80% of the maximum E2 induction, is run in quadruplicate
- The vehicle control (1% DMSO in EFM) is run in quadruplicate.
- All reference anti-estrogen and test wells contain a fixed concentration of E2 (9.18×10^{-11} M), intended to provide 80% of the maximum E2 induction.
- Tamoxifen, a weak antagonist reference standard, is plated in quadruplicate at 3.36×10^{-6}M.

2.7.2.2 Plate Design

- All 96 wells of the test plate are used during comprehensive testing, thereby allowing two substances tested at eleven concentrations in triplicate on each plate using starting concentrations that were determined during range finder testing **(Section 2.6.2**). The plate design for comprehensive antagonist testing is provided below in **Figure 2-7.**

Figure 2-7 Plate Layout for Comprehensive Antagonist Testing

TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS1-1	TS1-2	TS1-3	TS1-4	TS1-5	TS1-6	TS1-7	TS1-8	TS1-9	TS1-10	TS1-11	VC
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	VC
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Tam
TS2-1	TS2-2	TS2-3	TS2-4	TS2-5	TS2-6	TS2-7	TS2-8	TS2-9	TS2-10	TS2-11	Tam
Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam
Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam

Abbreviations: E2 = E2 control; Ral-1 to Ral-9 = concentrations of the Raloxifene/E2 reference standard (from high to low); Tam = Tamoxifen/E2 weak positive control; TS1-1 to TS1-11 = concentrations (from high to low) of test substance 1 (TS1); TS2-1 to TS2-11 = concentrations (from high to low) of test substance 2 (TS2); VC = vehicle control (DMSO [1% v/v EFM.]).

Note: As noted, all reference and test wells contain a fixed concentration of E2 (4.90×10^{-11}M)

2.7.2.3 Plate Acceptance Criteria for Comprehensive Antagonist Testing

- The mean DMSO control RLU values must be within 2.5 times the standard deviation of the historical DMSO control mean RLU value.
- Ral reduction must be greater than three-fold. Reduction is calculated by dividing the averaged highest Ral reference RLU value by the averaged lowest Ral RLU value.
- The Ral reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve.
- The averaged E2 control RLU value must be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- The mean plate tamoxifen control RLU value must be less than the mean E2 control RLU value minus three times the SD.

Following Phase 2a of the validation study, the failure rates of plates used to comprehensively test four antagonist substances were evaluated. The percentages of antagonist test plates that failed test plate acceptance criteria across the participating laboratories was 38% (13/34) (see **Section 7, Table 7-7).** To determine if changes to test plate acceptance criteria could reduce the failure rates of comprehensive test plates without compromising the ability of the test method to detect and quantify test substance antagonist activity, a comparison was made of qualitative (i.e., positive or negative antagonist classification) and quantitative (i.e., IC_{50} value) outcomes for test plates that met all acceptance criteria versus those that failed to meet one or more criterion (see **Section 7, Tables 7-8** and **7-9)**. Test plate acceptance criteria

based on the DMSO control RLU values and the Ral reference standard minimum fold-reduction values were not considered for modification because they are essential for monitoring background activity and reference antagonist performance. In addition, the E2 control test plate acceptance criterion was not considered for modification, because it is essential for determining test substance antagonist activity. Therefore, the test plate acceptance criteria that were considered for modification were the Ral IC_{50} and flavone control RLU values. Based on this evaluation, it was determined that antagonist test plate acceptance criteria could be modified without compromising the ability of the test method to detect and quantify test substance agonist or antagonist activity. These modifications were as follows:

- The requirement that the mean plate Ral reference standard IC_{50} value must be within 2.5 times the SD of the historical mean IC_{50} value was eliminated and replaced with a requirement that the Ral reference standard curve should be sigmoidal in shape and have at least three values within the linear portion of the curve
- The requirement that the mean plate flavone control RLU value must be within 2.5 times the SD of the historical mean flavone control RLU value was changed such that the flavone control RLU value must be less than three times the SD of the mean plate RLU value of the flavone control.

Changes to the antagonist test plate acceptance criteria described above were used for Phase 2b, 3, and 4 testing. However, as detailed in **Section 2.4.5,** further evaluation of the data after the study was completed led to the replacement of flavone with tamoxifen as the weak positive control for ER antagonism.

2.7.2.4 Interpretation of Results from Comprehensive Antagonist Testing

As described in **Section 2.7.2.1**, criteria used to classify substances as Positive or Negative for ER agonism or antagonism were modified following a retrospective analysis of the data. These new classification criteria, provided above, were applied to all test data for the assessment of test method accuracy (**Section 5**).

POSITIVE CLASSIFICATION

- Positive classification of ER antagonist activity should have a concentration response curve consisting of a baseline, followed by a negative slope.
- The line defining the negative slope must contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau.

- A positive classification requires a response amplitude, the difference between baseline and bottom, of at least 20% of the maximal value for the reference estrogen (i.e. 2000 RLU when the maximal response value of the reference estrogen is adjusted to 10,000 RLUs).
- The highest non-cytotoxic concentrations of the test substance should be less than or equal to 1x10-5 M.

NEGATIVE CLASSIFICATION

- Test substances are classified as negative for antagonist activity when all data points are above the ED80 value (80% of the E2 response, or 8000 RLUs)

INADEQUATE

- Data that that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of activity

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